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(54) **METHOD OF PRETREATING SAMPLE**

(57) A pretreatment method for enhancing relativity and reaction specificity prior to the detection or determination of an ingredient, especially a microorganism ingredient, contained in a biosample; and a pretreating fluid therefor or a reagent for determination containing the pretreating fluid. The pretreating fluid(or reactive fluid), which is for the determination of an ingredient contained in a sample to be tested, comprises at least an organic

acid or a salt thereof. If preferably comprises: at least one member selected among surfactants and substances capable of reduction; and an organic acid or salt thereof. Treatments with the pretreating fluid were found to eliminate problems and the invention has thus been completed.

## Description

## Technical field

**[0001]** The present invention relates to a method for a pretreatment of a test sample used for detection and measurement of an ingredient contained in a biosample particularly a microorganism and a reaction reagent, which includes a pretreatment reagent used for this treatment, used for immunological measurement and is used for a so-called diagnostic drug for a clinical test.

## Background Art

**[0002]** In detection and measurement of a specific ingredient, which is contained in a biosample including, for example, blood, spinal fluid, seminal fluid, saliva, urine, stool, sputum, rhinorrhea, secreted liquid, sweat, and the like, it is frequently necessary to pretreat these samples to make detection and measurement of the specific ingredient easy.

As measures for this purpose, various methods for pretreatment have been proposed so far. For example, it has been known that for measurement of a core antigen of a virus, a method for breaking pallium of the virus by a surfactant has been known to expose a core protein for measurement (JP P1996-50133 A and JP P1999-108932 A).

**[0003]** In addition, for measurement of a soluble lipopolysaccharide derived from a bacterium such as Chlamidia, a combination of an anionic polysaccharide with the surfactant has been disclosed (JP P1997-127110 A).

**[0004]** A problem of the present invention is to provide, in detection and measurement of the ingredient contained in the biosample, particularly a microorganism ingredient, the method for pretreatment to enhance reactivity and reaction specificity, a liquid for pretreatment, or a reagent containing the same for measurement.

**[0005]** For example, in order to detect immunologically influenza virus contained in sputum or rhinorrhea collected from a patient, sputum or rhinorrhea was necessarily treated with the pretreatment liquid to expose the antigen. In such the biosample, a large amount of a substance disturbing the measurement is contained and, therefore, the pretreatment is required to enhance reactivity of a target substance for the measurement without a bad influence to the measurement.

## Disclosure of the Invention

**[0006]** As a result of intensive studies by the present inventors, we found that the problem of the present invention can be solved by treating with a liquid for pretreatment of a test sample (or reaction liquid) for measurement of the ingredient contained in the test sample and a pretreatment liquid containing at least an organic acid or a salt thereof, particularly a pretreatment liquid containing at least one members selected from a surfactant and a reducing agent, and an organic acid or a salt thereof, resulting in completion of the present invention.

**[0007]** The present invention includes:

1. A method for pretreatment of a test sample in measurement of a microorganism-related substance in the test sample, wherein the test sample treated by a pretreatment solution, which contains at least an organic acid or a salt thereof, for the test sample;
2. The method according to foregoing paragraph 1, wherein the related substance is at least a microorganism such as virus, Rickettsia, bacterium, or fungus or a specific component derived from these microorganisms;
3. The method according to foregoing paragraph 1 or 2, wherein the test sample is a biological sample or the sample derived from a biological sample;
4. The method according to foregoing paragraph 3, wherein the biological sample or the sample derived from the biological sample is rhinorrhea, pus, a waste liquid by washing a nasal cavity, a waste liquid by wiping the nasal cavity, a waste liquid by wiping a pharynx, or sputum;
5. The method according to any of foregoing paragraphs 1 to 4, wherein the pretreatment solution further contains a surfactant and/or a reducing substance;
6. The method according to foregoing paragraph 5, wherein the surfactant is used in one member or in combination of two or more members selected from an anionic surfactant, a nonionic surfactant, a cationic surfactant, and an amphoteric surfactant;
7. The method according to foregoing paragraphs 5, wherein the reducing substance is a reductive compound containing sulfur;
8. The method according to any of foregoing paragraphs 1 to 7, wherein the organic acid or the salt thereof is used in one member or in combination of two or more members selected from acetic acid, succinic acid, tartaric acid, citric acid, and a salt thereof;
9. A method for an immunological measurement comprising the immunological measurement of the microorganism-

ism-related substance in the test sample after pretreatment of the test sample with the method according to any of foregoing paragraphs 1 to 8;

10. A reaction reagent for an immunological measurement containing a reagent used for the method for pretreatment of the test sample according to any of foregoing paragraphs 1 to 8 as a constitutional element; and

11. The reaction reagent for the immunological measurement according to foregoing paragraph 10, wherein the reagent is a reaction liquid.

#### Best Mode for Carrying Out the Invention

**[0008]** In the invention, a microorganism-related substance contained in a test sample is exemplified by a microorganism such as virus, Rickettsia, bacterium or fungus, or a specific component derived from these microorganisms. These are measured immunologically, particularly preferable for measurement of an antigen or an antibody, and specifically preferable for measurement and detection of a virus antigen. A specific example of the test sample includes, for example, viruses such as herpes virus (HSV, CMV, ZVZ, EBV, HHV, and the like), influenza virus, human immunodeficiency virus (HIV), human adult T - cell leukemia virus (HTLV), hepatitis virus (HBV, HCV, HDV, and HGV), and also lesion viruses of such as a cold syndrome, a digestive system disease, a central nerve system disease, a respiratory system disease, hemorrhagic fever, and other various diseases. Especially, it is preferable for measurement of an influenza antigen. Not restricted to this, it can be used for measurement of the virus antigen necessary for a pretreatment to expose the antigen. Moreover, other than viruses, it can be applied to various microorganisms, for example, bacteria (Staphylococcus aureus, Escherichia coli, and Bacillus of green pus) and Chlamidia, which require the pretreatment for exposure of the antigen.

**[0009]** The test sample in the invention is the ingredient contained in a biosample or the sample derived from the biosample. The biosample or the sample derived from the biosample includes a body fluid such as whole blood, plasma, serum, urine, spinal fluid, seminal fluid, saliva, human milk, sweat, mucus; stool, a lesion tissue and its extract, pus, sputum, rhinorrhea, a waste liquid by washing a nasal cavity, the waste liquid by wiping the nasal cavity, the waste liquid by wiping a pharynx, a cultured sample of a microorganism such as virus, and the like. When the specific ingredient contained in the biosample is immunologically detected and measured, the test sample is previously treated with the pretreatment solution to subject to detecting and measuring reactions.

**[0010]** The organic acid or the salt thereof used in the invention is not specially restricted, and, for example, one member or a combination of two or more members, which are selected from acetic acid, succinic acid, tartaric acid, citric acid and a salt thereof, is used. The organic acid or the salt thereof, which is used in the invention, may be used singly or by blending two or more members of them. As other organic acids, oxalic acid, glycolic acid, gluconic acid, malic acid, and the like are exemplified.

**[0011]** The surfactant is used in one member or a combination of two or more members selected from an anionic surfactant, a nonionic surfactant, a cationic surfactant, or an amphoteric surfactant in the invention. The surfactant used is not specially restricted, and representative examples include polyoxyethylene alkyl phenyl ether, polyoxyethylene alkyl ether, polyoxyethylene sorbitan alkyl ester, alkyl pyridinium salt, higher alcohol sulfate ester salt, and the like.

**[0012]** The reductive substance used in the invention is a reducing compound containing sulfur and used singly or in blend of two or more members. A representative reductant includes reductive compounds containing sulfur, such as mercaptoethylamine, mercaptoethanol, dithiothreitol, cysteine, N-acetyl-L-cysteine, hydrodibromic acid S-2 aminoethylisothiouraea, tris (2-carboxyethyl) phosphine, a hydrosulfite salt, a sulfite, and the like.

**[0013]** An amount for use of these substances in pretreatment is determined as a concentration in a solution of the test sample. The added amount of the organic acids in total is a minimal 5 mM or higher, preferably ranges 10 to 500 mM, and more preferably ranges from 50 to 100 mM. Adding 100 mM or higher amount yields no special effect of the treatment, but the amount may be used. The added amount of the surfactant in total is 0.01 w/v% or larger, preferably 0.05 w/v% or larger, and an upper limit is 5 w/v%, preferably 1 w/v%, and more preferably 0.125 w/v%. Adding 0.125 w/v% or higher amount yields no special effect of the treatment, but the amount may be used. The added amount of the reductants in total is a minimal 0.5 mM or higher, preferably ranges 1 to 500 mM, and more preferably ranges from 5 to 50 mM. Adding 10 mM or higher amount yields no special effect of the treatment, but the amount may be used.

**[0014]** In an example of specific embodiment according to the invention, such a solution is used as the pretreatment solution that contains 0.01 to 5 w/v%, more preferably 0.05 to 1.0 w/v% of polyoxyethylene nonylphenyl ether (commercial name NP-40), which is the nonionic surfactant, is used as the surfactant, 1 to 100 mM, more preferably 10 to 50 mM of a hydrochloric acid salt of 2-mercaptoethylamine is used as the reductant, 10 to 500 mM, more preferably 50 to 100 mM of citric acid is used as the organic acid, and that has pH adjusted to 5 to 7, more preferably about 6.

**[0015]** The test sample in the invention is measured by immunochemical method following the pretreatment, for example, through blending 100  $\mu$ L of the pretreatment solution with a 20  $\mu$ L of a patient's rhinorrhea containing influenza virus. The method is particularly exemplified by sandwich enzyme immunossay method by using an anti-influenza monoclonal antibody or particle-labeling immunochromatographic method through labeling the anti-influenza mono-

clonal antibody with a colored latex particle, and the like.

#### Example

**[0016]** The invention will be described with examples below and the present invention is not restricted to these examples.

#### Example 1

**[0017]** Various surfactants were added to a 20 mM phosphate buffer solution (pH 6.0) to prepare pretreatment solutions. Following blending 100 $\mu$ L of each of the pretreatment solution with cultured influenza virus to treat at an ordinary temperature for 10 min, a virus antigen was measured by the enzyme immunossay method by using the anti-influenza virus monoclonal antibody. The result will be presented in Table 1.

Table 1

Effect of pretreatment for influenza virus measurement using various surfactants					
(Absorbency at 492 nm)					
Surfactant	Concentration (W/V%)				
	0	0.06	0.125	0.25	0.5
Nonidet P-40	0.101	0.788	0.762	0.758	0.839
Triton X-100	0.101	0.710	0.748	0.725	0.733
Tween 80	0.101	0.169	0.196	0.216	0.267
Tween 20	0.101	0.623	0.606	0.562	0.580
Nonion HS-210	0.101	0.778	0.783	0.749	0.644
Nonion HS-240	0.101	0.129	0.132	0.125	0.131
Nonion A10-R	0.101	0.849	0.819	0.816	0.875
Emergen 909	0.101	0.753	0.757	0.771	0.754
Bridge 35	0.101	0.531	0.533	0.527	0.573
Bridge 58	0.101	0.095	0.322	0.415	0.396
Bridge 76	0.101	0.675	0.686	0.652	0.725
Bridge 97	0.101	0.713	0.730	0.687	0.729
Bridge 98	0.101	0.588	0.341	0.598	0.530
Bridge 721	0.101	0.135	0.313	0.403	0.391
CHAPS	0.101	0.125	0.161	0.383	0.552
CHAPSO	0.101	0.132	0.209	0.477	0.541
Octylglucoside	0.101	0.115	0.111	0.117	0.585
Octylthioglucoside	0.101	0.128	0.142	0.615	0.840

**[0018]** As the result of the above experiment, all examined surfactants showed that intensity of a measurement signal in the enzyme immunossay method enhances in a concentration range at least from 0.06 to 0.5 w/v%, which expresses clearly the effect of the pretreatment.

#### Example 2

**[0019]** Except for addition of various reductants to the 20 mM phosphate buffer solution containing 0.1 w/v% Nonidet P-40, the operation was conducted in the same way as that in Example 1 to test the effect of reductants. The result will be presented in Table 2.

Table 2

Effect of pretreatment by various reductants				
(Absorbency at 492 nm)				
Reluctant	Concentration (mM)			
	0	2	10	50
N-acetyl-L-cysteine	0.129	0.445	0.916	0.998
Hydrodibromic acid S-2 aminoethylisothiurea	0.129	1.021	1.209	1.411
2-mercaptoethylamine hydrochloride	0.129	1.201	1.505	1.554
Tris (2-carboxyethyl) phosphin	0.129	0.372	0.674	0.902
Dithiothreitol	0.129	0.860	1.010	0.908

[0020] As the result of the above experiment, it was found that by adding reductants, a larger absorbency was observed and a large effect of the pretreatment was yielded.

#### Example 3

[0021] Except for addition of various organic acids to the 20 mM phosphate buffer solution containing 0.1 w/v% Nonidet P-40 and 20 mM 2-mercaptoethylamine hydrochloride, the operation was conducted in the same way as that in Example 1 to test the effect of organic acids. The result will be presented in Table 3.

Table 3

The effect of organic acids					
(Absorbency at 492 nm)					
	Concentration (mM)				
	0	10	50	100	200
Citric acid	0.125	0.325	0.415	0.422	0.425
Succinic acid	0.125	0.154	0.189	0.203	0.204
Acetic acid	0.125	0.204	0.216	0.243	0.245
Oxalic acid	0.125	0.168	0.199	0.211	0.209

[0022] As the result of the above experiment, it was found that by adding organic acids, a larger signal was observed and the effect of the pretreatment was high.

#### Example 4

[0023] The following pretreatment solution was prepared: the 20 mM phosphate buffer solution (pH 6.0, 0.1% ONP/NaPB) containing 0.1 w/v% Nonidet P-40; a solution (NP40+NAC/NaPB) prepared by adding 10 mM N-acetyl-L-cysteine to 20 mM phosphate buffer solution (pH 6.0) containing 0.1 w/v% Nonidet P-40; a solution (NP40+NAC/citrate) prepared by adding 10 mM N-acetyl-L-cysteine to 100 mM citric acid buffer solution (pH 6.0) containing 0.1 w/v% Nonidet P-40; and a solution (NP40+MEA/citrate) prepared by adding 10 mM 2-mercaptoethylamine hydrochloride to 100 mM citric acid buffer solution (pH 6.0) containing 0.1 w/v% Nonidet P-40. Then, each sample of rhinorrhea or the waste liquid by wiping the pharynx (No. 5x2, No. 10, No. 19, No. 20, and No. 31) of the influenza patient, the cultured virus antigen (NIBSC Corp. made), and a commercial influenza antigen (A/Texas/77 Chemicon Corp. made) was pretreated and then, an influenza antigen was measured by the immunoassay method using the anti-influenza virus monoclonal antibody. The result will be presented in Table 4.

Table 4

The result of measurement of the influenza antigen using various pretreatment solutions							
(Absorbency at 492 nm)							
Pretreatment solution	No.5 × 2	No. 10	No. 19	No. 20	No. 31	Sydney	CHEMICON
0.1%NP40/NaPB	0.29	0.036	0.002	0.224	0.135	0.485	0.089
NP40+NAC/NaPB	0.330	0.079	0.034	0.254	0.157	0.554	0.418
NP40+NAC/Citrate	0.386	0.094	0.036	0.423	0.25	0.798	0.808
NP40+AET/Citrate	0.448	0.09	0.053	0.466	0.193	0.78	0.861
NP40+MEA/Citrate	0.434	0.095	0.064	0.435	0.235	0.779	1.224

[0024] From the result as described above, it was found that in comparison with the sample treated with the pretreatment solution of the 20 mM phosphate buffer solution (pH 6.0, 0.1% NP/NaPB), which contains 0.1 w/v% Nonidet P-40, and the pretreatment solution, to which the reductant NAC was added, the sample treated with the pretreatment solution, to which the organic acid was added, yielded the larger signal and the effect of the pretreatment was high.

#### Effect of the Invention

[0025] Through a treatment with the liquid for pretreatment of the test sample (or reaction liquid) for measurement of the component contained in the test sample and the pretreatment liquid containing at least the organic acid or the salt thereof, particularly the pretreatment liquid containing at least one selected from the surfactant and the reducing agent, and the organic acid or the salt thereof, reactivity and reaction specificity are enhanced in detection and measurement of an ingredient, particularly a microorganism component, contained in a biosample

#### Claims

1. A method for pretreatment of a test sample in measurement of a microorganism-related substance in the test sample, wherein the test sample treated by a pretreatment solution containing at least an organic acid or a salt thereof.
2. The method according to claim 1, wherein the microorganism-related substance is at least a microorganism such as virus, Rickettsia, bacterium or fungus, or a specific ingredient derived from these microorganisms.
3. The method according to claim 1 or 2, wherein the test sample is a biological sample or the sample derived from a biological sample.
4. The method according to claim 3, wherein the biological sample or the sample derived the biological sample is rhinorrhea, pus, a waste liquid by washing a nasal cavity, the waste liquid by wiping the nasal cavity, the waste liquid by wiping a pharynx, or sputum.
5. The method according to any of claims 1 to 4, wherein the pretreatment solution contains a surfactant and/or a reducing substance.
6. The method according to claim 5, wherein the surfactant is used in one member or in combination of two or more members that are selected from an anionic surfactant, a nonionic surfactant, a cationic surfactant, and an amphoteric surfactant.
7. The method according to claim 5, wherein the reducing substance is a reductive compound containing sulfur.
8. The method according to any of claims 1 to 7, wherein the organic acid or the salt thereof is used in one member or in combination of two or more members selected from acetic acid, succinic acid, tartaric acid, citric acid, and the salt thereof.
9. A method for an immunological measurement comprising the immunological measurement of the microorganism-

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related substance in the test sample after pretreatment of the test sample with the method according to any of claims 1 to 8.

5 10. A reaction reagent for an immunological measurement containing a reagent used for the method for pretreatment of the test sample according to any of claims 1 to 8 as a constitutional element.

11. The reaction reagent for the immunological measurement according to claim 10, wherein the reagent is a reaction liquid.

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP01/06589

A. CLASSIFICATION OF SUBJECT MATTER  
Int. Cl.<sup>7</sup> G01N33/48

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
Int. Cl.<sup>7</sup> G01N33/48Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
Jitsuyo Shinan Koho 1922-1996 Toroku Jitsuyo Shinan Koho 1994-2001  
Kokai Jitsuyo Shinan Koho 1971-2001 Jitsuyo Shinan Toroku Koho 1996-2001

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 8-313525 A (Konica Corporation), 29 November, 1996 (29.11.96), (Family: none)	1-4 8-11
Y		5-7
Y	JP 9-501494 A (Quidel Corporation), 10 February, 1997 (10.02.97), & EP 712495 A & WO 95/004280 A & US 5415994 A	1-11
Y	JP 2000-206115 A (Eiken Chemical Co., Ltd.), 28 July, 2000 (28.07.00), (Family: none)	5, 7
Y	JP 6-324040 A (Konica Corporation), 25 November, 1994 (25.11.94), (Family: none)	5, 7

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

\* Special categories of cited documents:  
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 "&" document member of the same patent family

Date of the actual completion of the international search  
23 October, 2001 (23.10.01)Date of mailing of the international search report  
30 October, 2001 (30.10.01)Name and mailing address of the ISA/  
Japanese Patent Office

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